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Cancer Immunol Immunother. 1990;32(3):185-90.

Cancer Immunol Immunother. 1990;31(4):231-5.

Cancer Immunol Immunother. 1992;35(3):158-64.

Med Oncol Tumor Pharmacother. 1993;10(1-2):61-70.

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Int J Pancreatol. 1997 Feb;21(1):43-52.

Hybridoma. 1988 Oct;7(5):429-40.

Jpn J Cancer Res. 1990 Mar;81(3):266-71.

Christopher Yaen
US Patent Office
Art Unit 1642
571-272-0838
REM 3A20
REM 3C18

HYBRIDOMA
Volume 7, Number 5, 1988
Mary Ann Liebert, Inc., Publishers

Effect of Human Blood Mononuclear Cell Populations in Antibody Dependent Cellular Cytotoxicity (ADCC) Using Two Murine (CO17-1A and Br55-2) and One Chimeric (17-1A) Monoclonal Antibodies Against a Human Colorectal Carcinoma Cell Line (SW948)

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ABSTRACT

Peripheral blood mononuclear cells (PBMC) from healthy individuals were studied for their lytic capability in ADCC using SW948 (a human colorectal carcinoma cell line) as target cells. Three monoclonal antibodies (MAbs) were used: two mouse MAbs (IgG_{2A}) against the antigenic structures CO17-1A and BR55-2 respectively and one chimeric MAb 17-1A (IgG_1) (mouse-human). Three kinds of effector cells were prepared. PBMC were purified on a Ficoll-Isopaque gradient (FIP cells) (a mixture of lymphocytes and monocytes). To obtain pure monocytes (>90%), PBMC were centrifuged on a Nycodenz gradient (Nycodenz cells). Highly purified lymphocytes (>98%) were obtained by treatment of FIP cells with iron powder and removal of phagocytic cells (PBL cells). Monocytes had the highest lytic capability. FIP cells were less effective than monocytes. PBL cells had the poorest killing activity. In reconstitution experiments addition of increasing amount of monocytes to PBL resulted in an augmented cytotoxicity. The numbers of Leu-M3$^+$ cells, Leu-M5$^+$ cells (monocytes) and CD16$^+$ cells correlated positively to cytotoxicity. Higher concentration of MAb 17-1A was required to reach the same level of cytotoxicity using FIP cells as effector cells as compared to monocytes. MAb BR55-2 induced the same cytotoxic activity as MAb 17-1A. Combination of these two MAbs did not increase the lytic capability. Chimeric MAb 17-1A mediated ADCC in a dose-dependent fashion. The chimeric MAb was consistently more effective than the mouse MAb.

INTRODUCTION

The availability of monoclonal antibodies (MAbs) has broadened the immunological approach to the study of human cancer. Since the original publication on the production of murine monoclonal antibodies (1), MAbs have been widely applied for characterization of tumor associated antigens (TAA), for diagnostic purposes as well as for passive serotherapy (2).

MAb 17-1A was one of the first MAbs used for diagnotic imaging technique

and also for therapy of humans (3). In animal and in vitro experimental systems, antibody dependent cellular cytotoxicity (ADCC) has been claimed to be an important mechanism for tumor cell destruction (4,5). In vitro, the main mononuclear cell subpopulations responsible for 4h ADCC are NK/K cells bearing Fc receptors and in long term ADCC (>16-18h) also monocytes/macrophages (6,7,8). Monocyte enriched cells depleted of $CD16^+$ cells exerted ADCC activity in the presence of mouse MAb 17-1A (IgG_{2a}) or chimeric (mouse x human) MAb 17-1A (IgG_1) (9). Post-treatment tumor biopsies from nude mice transplanted with fresh human tumors or human cancer cell lines and treated with intraperitoneal injections of MAb 17-1A showed infiltration of activated macrophages which exhibited a specific ADCC activity (6,10). In a mouse melanoma system, tumor cell lysis of MAb treated mice was mediated by NK cells (8). In the majority of patients with metastatic colorectal carcinomas treated with MAb 17-1A, an increased number of monocytes and NK cells and sometimes also of T cells were noted in the tumor after infusion of MAb 17-1A (11). In an ADCC system using a human colorectal carcinoma cell line (SW948), MAb 17-1A and blood mononuclear cells from patients with metastatic colorectal carcinoma, a significant positive correlation was noted between the numbers of $Leu\text{-}M5^+$ (monocyte marker) cells and ADCC activity. Moreover, higher cytotoxicity was also seen by increasing numbers of $CD16^+$ cells (12).

After infusion of unconjugated MAbs in cancer patients, effector functions are activated to destroy the tumor cells. To achieve a successful therapeutic result, it is important to have detailed information on the complex effector system as a basis for future improvements, especially with regard to the possibility to selectively activate various immune functions. It is important to have knowledge on the type of effector cells involved in the lytic process, the optimal MAb concentration and interaction between MAbs recognizing different TAAs on the tumor cells. In this study we have analyzed blood mononuclear cell subsets and their relative contribution to the lytic capability in ADCC using three different MAbs against the human colorectal carcinoma cell line SW948.

MATERIAL AND METHODS

Human Effector Cells

Heparinized venous blood from healthy, adult donors were separated by centrifugation on a Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) gradient (density:1.077 g/ml) (FIP cells) (13). EDTA (0.002%) blood was separated on a Nycodenz (density:1.006 g/ml) gradient (Nyegaard A/S, Oslo, Norway) to obtain enriched monocytes (Nycodenz cells) (14). To obtain purified lymphocytes, FIP cells were incubated with iron powder for 30 minutes at 37°C and phagocytic cells were removed by a magnet (PBL cells) (15).

Surface Markers

Isolated blood mononuclear cells were analysed for subpopulations in indirect immunofluorescence (IFL) using an absorbed goat anti-mouse IgG antibody in the second step (Meloy Lab., Springfield, VA, USA; B-D, Mountain View, CA, USA). Before staining, the cells were incubated at 37°C for 30 min. and washed twice in Hank-Tris solution (HT). The method has been described in detail elsewhere (16). MAbs used for staining are listed in Table 1. Two to four hundred cells were counted in a Leitz Dialux 20 B with epiillumination in ultraviolet light x 1000 magnification or by flow cytometry (FACScan B-D Moutain View, Ca, USA) analysing 10^4 cells at 480 nm with a flow rate of <300 cells/s.

Antibody Dependent Cellular Cytotoxicity (ADCC).

The target cells used were SW948, a human colorectal cancer cell line, expres-

MAbs Used for

MAb
OKT4[a], Leu-3a[b]
OKT8[a], Leu-2a[b]
OKM1[a]
Leu-M1[b]
Leu-M3[b]
Leu-M5[b]
Leu-7[b]
Leu-11[b]

a= Ortho-Pharm
b= Becton Dick

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$$\% \text{ lysis} = \frac{\text{rel}}{\text{max}}$$

One lytic unit
obtain 30% spe

TABLE 1.

MAbs Used for Staining

MAb	CD number	Relevant specificity
OKT4[a], Leu-3a[b]	CD4	T-helper/inducer
OKT8[a], Leu-2a[b]	CD8	T-suppressor/cytotoxic
OKM1[a]	CD11	Monocytes/macrophages
Leu-M1[b]	CD15	Monocytes/granulocytes
Leu-M3[b]	-	Monocytes/macrophages
Leu-M5[b]	-	Monocytes/macrophages
Leu-7[b]	-	NK cells/T cells
Leu-11[b]	CD16	NK cells/neutrophils

a= Ortho-Pharmaceutical, Raritan, NJ, USA
b= Becton Dickinson, Mountain View, CA, USA

sing the antigen CO17-1A. After trypsinization of the cells and test for viability (Trypan blue) the cells were labelled with 2.8 MBq sodium chromate-^{51}Cr (spec. act. 9-18.5 GBq/mg chromium, Radiochemical Centre, Amersham, England) per ml of cells at 37°C for 1h. After washing three times in full medium (Leibovitz medium 15, Gibco Ltd, Scotland, containing 10% FCS, antibiotics and 2 mmol l-glutamine) the cells were added to round-bottom wells of a 96 micro-titer plate (Nunc, Roskilde, Denmark) and effector cells added given effector to target cell ratios of 100, 50, 25, 12, 6. Antibody was added at the concentrations required by the experimental design. Effector cells and target cells without antibody were used as negative controls. The percentage of ^{51}Cr release of 20 negative controls at the above mentioned E:T ratios was 10 ± 3, 8 ± 3, 3 ± 2, 2 ± 2, 1 ± 1 (mean ± SD). Moreover, as a further negative control a non relevant MAb, F1, (a mouse IgG_{2a} MAb against an antigenic structure on CLL cells) was included. The presence of F1 in the assay did not mediate any cytotoxicity against SW948. The final incubation volume was 0.2 ml. Each run was done in triplicate. The assay mixtures were incubated at 37°C for 18h in humified air with 5% CO_2 (12,17,18). Maximal ^{51}Cr release from the target cells was determined after treatment with Triton-X100 (Merck, Darmstadt, West-Germany). Spontaneous release was to 25-30%. The radioactivity of supernatant was measured in a gamma counter. Per cent cytotoxicity was calculated by the following formula:

$$\% \text{ lysis} = \frac{\text{release in sample - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

One lytic unit was defined as the number of mononuclear cells required to obtain 30% specific lysis.

MAbs Against TAA on SW948

Two mouse MAbs, 17-1A (IgG_{2a}-K) and BR55-2 (IgG_{2a}-λ)(4,19) and a mouse-human chimeric MAb 17-1A (IgG_1-K)(20,21) were used.

Statistics

Analyses of differences between means were done by Student' s t-test. The linear regression model was used to estimate correlation between independent observations.

RESULTS

Surface Markers

The percentage of blood mononuclear cell subpopulations obtained by FIP gradient centrifugation is shown in Table 2. The majority of the cells was of T cell origin. About 10-15% expressed NK cell markers and about 20% was of monocyte origin. In the monocyte enriched cell fraction (Nycodenz cells), >90% of the cells expressed monocyte related antigens. Very few T cells were present. When phagocytic cells were removed by iron powder from the FIP cell fraction, few monocytes remained and $CD16^+$ cells also decreased.

TABLE 2

Cell Subpopulations (%) (mean±SE) Obtained by the Different Purification Procedures

Surface marker	FIP cells	NYCODENZ cells	PBL cells
CD11	13±3 (13)[a]	93±1 (8)	6±2 (4)
LEU-M5	22±2 (4)	95±1 (8)	5±1 (4)
LEU-M3	25±3 (5)	79±2 (8)	3±1 (4)
CD15	3±2 (2)	42±1 (8)	<0.25 (4)
LEU-7	17±2 (12)	1±2 (3)	2±4 (4)
CD16	10±2 (13)	7±2 (4)	4±2 (3)
CD4	42±2 (13)	1±0.1 (4)	57±1 (4)
CD8	31±1 (13)	0.5±0.1 (4)	31±1 (4)

a. Number of tests.

Murine MAb 17-1A in ADCC

The ADCC activity induced by MAb 17-1A using various mononuclear cell fractions is shown in Fig 1. The monocyte enriched cell fraction exerted the strongest cytotoxicity. In one representative experiment shown in Fig. 1, the

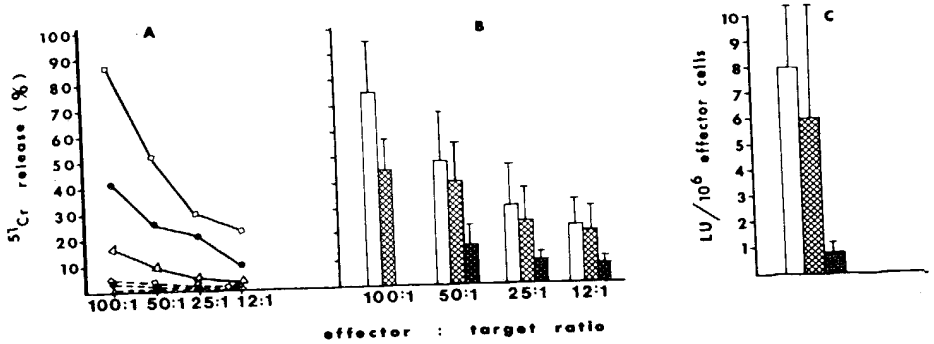

Fig.1 ADCC using SW948 as target cell and MAb 17-1A (10^{-3} mg/ml). A: (□) Nycodenz cells; (●) FIP cells; (△)PBL cells. (⟵) in the presence of MAb 17-1A; (----) no MAb (one exp.). B: ADCC (%) (mean±SD) of Nycodenz cells (□)(n=8), FIP cells (▩) (n=13) and PBL cells (■)(n=4). (Significance levels: □ vs ▩ n.s; □ vs ▩ $p<0.01$; ▩ vs ■ $p<0.01$ for ratios 50:1; 25:1, 12:1. C: ADCC (LU/10^6 cells)(mean±SD)(see Fig. 1B)(Significance levels: □ vs ▩ n.s; □ vs ■ $p<0.01$; ▩ vs ■ $p<0.01$).

monocyte enriched cells induced a two fold increase in cytotoxic activity compared to FIP purified cells. PBL cells gave very low cytotoxicity. In a series of experiments monocyte enriched cells exerted the strongest cytotoxicity but not significantly different from FIP cells (Figs. 1B and C). PBL had the weakest killer activity. Cytotoxicity expressed in lytic units (LU) was more than six times higher in FIP and Nycodenz separated cells respectively compared to PBL cells (Fig. 1C).

Correlation between Mononuclear Cell Phenotypes and Cytotoxicity

To analyse cell subsets of importance participating in the tumor cell lysis, the cytotoxicity was plotted against the relative frequency of various cell populations. There was a significant positive correlation between $CD16^+$, $Leu\text{-}M3^+$ and $Leu\text{-}M5^+$ cells respectively and the percent cytotoxicity (Figs. 2A, B and C). No significant correlation between the other phenotypic cell markers and cytotoxicity was found.

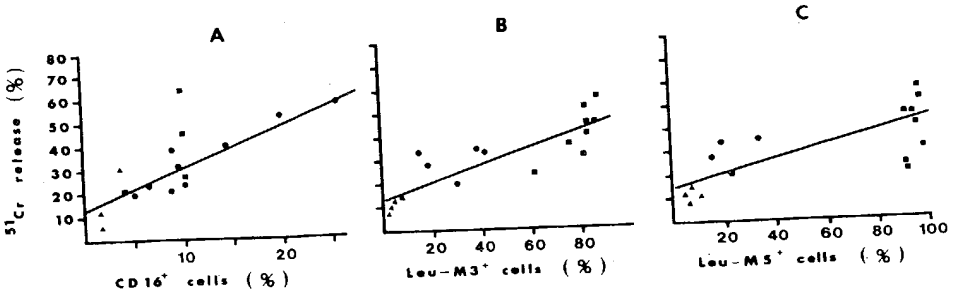

Fig.2 Relationship between the percentage of $CD16^+$ cells (A)($r=0.64$, $p<0.01$, $n=17$), $Leu\text{-}M3^+$ cells (B)($r=0.84$, $p<0.01$, $n=17$) $Leu\text{-}M5^+$ (C)($r=0.71$, $p<0.01$, $n=16$) and ADCC activity. MAb 17-1A conc. 10^{-3} mg/ml. E:T ratio 25:1. FIP cells (●) Nycodenz cells (■); PBL cells (▲).

Recombination Experiments

To analyze the relative contribution of monocytes and lymphocytes respectively in ADCC, recombination experiments were performed (Fig. 3). The lytic activity

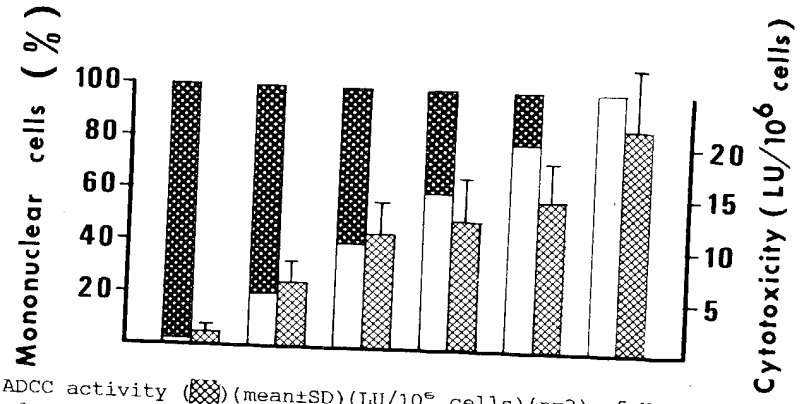

Fig.3 ADCC activity (▨)(mean±SD)(LU/10^6 cells)(n=3) of Nycodenz cells (□) and PBL cells (▩). Nycodenz cells and PBL cells were mixed in different proportions before adding to the target cells. MAb 17-1A conc. 10^{-3} mg/ml.

increased in parallel with increasing numbers of monocytes. The monocyte fraction showed a cytotoxic activity of 22 LU/10^6 cells, which was almost 10 times higher than that of PBL cells (2 LU/10^6 cells).

Titration of Murine MAb 17-1A and MAb BR55-2

Increasing concentrations of MAb 17-1A augmented the cytotoxic potential of FIP cells (Fig. 4A). 10^{-2} mg/ml of MAb induced a 80% killing while ten times diluted MAb exerted 40% cytotoxicity at an effector to target cell ratio of 50:1. MAb BR55-2, which recognizes an other antigen on the same target cell, also mediated a stronger cytotoxicity at 10^{-2} mg/ml than at 10^{-3} mg/ml (Fig. 4B). Mixing equal proportions of the MAbs did not induce a synergistic cytotoxic activity either at a concentration of 10^{-2} or at 10^{-3} mg/ml (Fig. 4C).

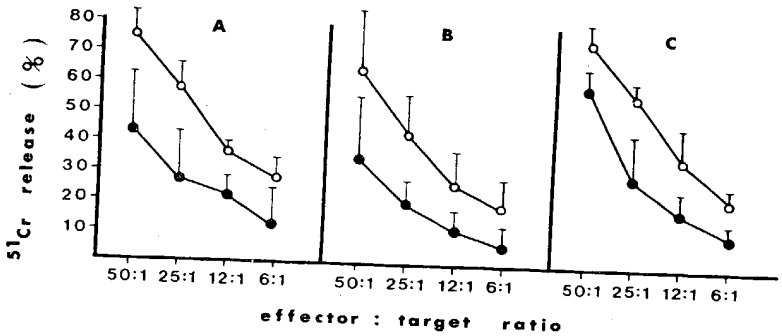

Fig.4 ADCC activity (%)(mean±SD)(n=3) of FIP cells. MAb conc.: 10^{-2} mg/ml (○) and 10^{-3} mg/ml (●). MAb 17-1A alone (A), BR55-2 alone (B) and a mixture in equal (1:1) proportions of both of them (C).

Monocyte enriched cell populations induced the same level of cytotoxic activity both at a concentration of 10^{-2} mg/ml and at 10^{-3} mg/ml of MAb 17-1A (Fig.

5A). The same was noticed for MAb BR55-2 alone (Fig. 5B) or when mixing MAb 17-1A and MAb BR55-2 in equal proportions (Fig. 5C).

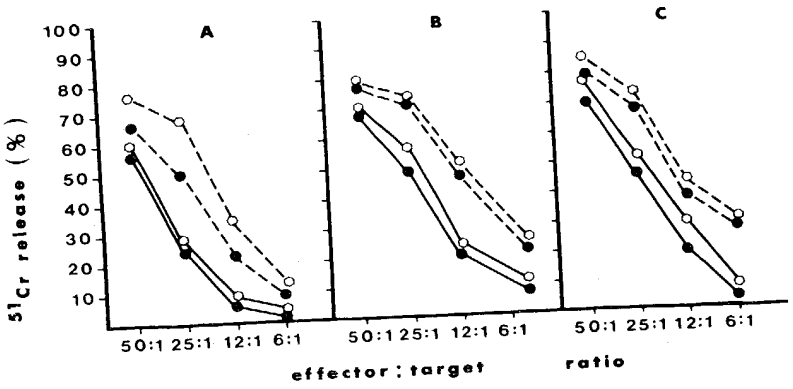

Fig. 5 ADCC activity (%) of Nycodenz cells using two concentrations of MAb. Exp 1 (——); exp 2 (---). For symbols see Fig. 4.

Fig. 6 summarizes the effect of different concentrations of MAbs using FIP cells and Nycodenz cells respectively. MAb 17-1A alone, BR55-2 alone or a mixture of both of them showed no significant difference in recruiting cytotoxic cells at the two concentrations studied. A ten fold increase in the concentration of MAb induced a 2.5 times increase in lytic activity using FIP cells, which effect was less pronounced for Nycodenz cells.

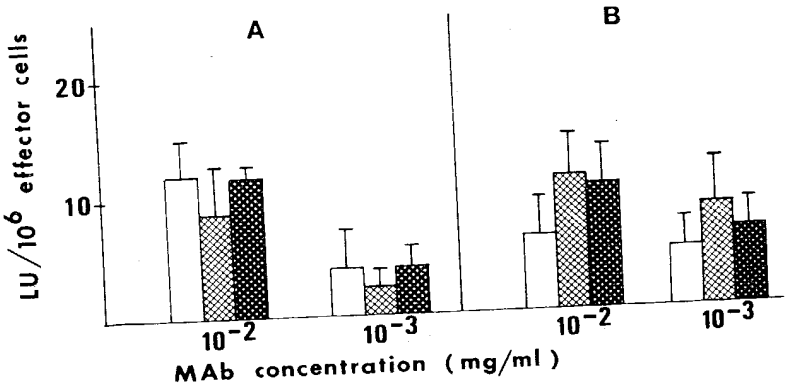

Fig.6 ADCC activity (LU/10^6 cells)(mean±SD)(n=4) of FIP cells (A) and Nycodenz cells (B) using MAb 17-1A alone (□), MAb BR55-2 alone (▨) and a mixture of both of them in equal proportions (▩).

Chimeric MAb 17-1A in ADCC

To avoid the generation of anti-mouse constant Ig region antibodies in patients treated with murine MAb 17-1A, a chimeric MAb 17-1A was produced (19). The chimeric MAb is composed of the V-regions of the mouse MAb while the

remaining protein of the Ig molecule is human. Increasing concentrations of the chimeric MAb augmented significantly the cytolytic activity (Fig. 7). 10^{-2} mg/ml induced a six fold higher lytic activity than 10^{-3} mg/ml while the effect of a higher concentration (10^{-1} mg/ml) was less pronounced, probably reaching a plateau level. The chimeric MAb 17-1A was highly statistically significantly more effective in ADCC than the mouse MAb 17-1A as shown in Table 3.

TABLE 3

ADCC (LU/10^6 cells) mediated by a mouse MAb 17-1A and a chimeric MAb 17-1A using FIP cells.

Exp.	MONOCLONAL ANTIBODIES	
	Mouse 17-1A*	Chimeric 17-1A*
I	0.7	
II	0.8	1
III	2	1.6
IV	1	3.5
V	0.6	4
VI	0.2	3.6
		2.5
Mean ± SD	0.9 ± 0.6	2.68 ± 1.1**

* Conc. 10^{-3} mg/ml.

** Significance level: $p < 0.01$, t-value = 4.61 (paired t-test).

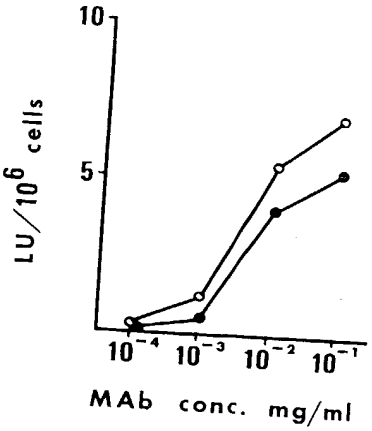

Fig.7 ADCC of FIP cells using chimeric MAb 17-1A (○) and mouse MAb 17-1A (●). (One representative experiment).

DISCUSSION

Monocyte enriched cells obtained by Nycodenz gradient separation mediated the strongest cytotoxic activity in a 18 h ADCC assay. The cytotoxic capability of

FIP purified cells was lower although statistically not different from that of the monocyte enriched population. Highly purified lymphocytes exerted the lowest lytic activity. A positive correlation between the numbers of CD16$^+$, Leu-M3$^+$ and Leu-M5$^+$ cells respectively and cytotoxicity was found which has also been noted previously (12). It has been shown in mice and in humans that both lymphocytes and monocytes/macrophages are strongly implicated as effector cells in 18 h ADCC (4,5,7,9). Mouse IgG_{2a} induced the highest lytic activity while IgG_3, IgG_1 and IgG_{2b} was less effective in that order (9). Short time (4 h) ADCC is mainly mediated by Fc-receptor bearing NK/K cells and induced preferably by MAbs of IgG_{2b} and IgG_3 subclasses (8,22). The relative contribution of monocytes compared to NK cells in our system (18h ADCC) was not fully analysed and needs further studies using highly purified cell subsets. However, monocyte enriched cell fractions had the highest cytolytic activity but removal of phagocytic cells did not completely eliminate the killing capacity. Lymphocyte enriched population expressed a low but detectable lytic activity, which might be due to the presence of a small fraction of Leu-M^{3+}, Leu-M5$^+$ and CD16$^+$ cells. Moreover, monocyte enriched fraction restored the lytic activity of a mononuclear cell population deprived of phagocytic cells.

The role of monocyte subsets expressing various antigenic markers (Leu-M1, M3 and M5) is not known . These markers may detect monocyte populations of various maturational steps (N. Warner, personal communication). It is interesting to note that in this and in another study (12), we found a positive correlation between the numbers of Leu-M5$^+$ cells and cytotoxicity. Moreover, in biopsy material from patients with metastatic colorectal carcinomas treated with MAb 17-1A, we noticed an increase in Leu-M5$^+$ cells after infusion of MAb 17-1A. The cells were localized close to the basement membrane of the tumor gland where MAb 17-1A was detected (11).

A ten fold increase in the concentration of MAb augmented the killing capacity of FIP cells by 50%. Using monocyte enriched cells, a ten fold increase in the MAb 17-1A concentration did not induce any appreciable enhancement of the cytotoxicity. Already at a concentration of 10^{-3} mg/ml of the MAb, the system was saturated and expressed maximal activity. This is in agreement with Johnson et al. (7) who described in the mouse the interaction between murine macrophages, tumor cells and MAb 17-1A and found that the lytic process required 24-48h of interaction between macrophages and tumor cells. $5x10^{-3}$ mg/ml of MAb induced maximum cytotoxic effect. 150.000 molecules of MAb 17-1A was bound to the target cell (SW-1116) and only this subclass could mediate killing in that particular system.

MAb BR55-2, which recognizes a different molecule on the same target cells (6), had a similar cytotoxic capability as MAb 17-1A. Combination of the two MAbs did not augment the killing capability. This may be taken into account in the therapeutic situation. Provided that all tumor cells express the relevant antigen, combination of MAbs might not be necessary but increasing doses of the appropriate MAb seem more likely to facilitate tumor lysis. However, if all tumor cells not express the relevant target structure, then combination MAbs should be necessary to achieve maximum tumor lysis.

Repeated therapy with MAb induces anti-mouse antibodies and might render the treatment ineffective (23). To avoid this problem, chimeric MAb against the antigen CO17-1A has been produced by genetic engineering (20,21). The killing mediated by the chimeric MAb was higher than that mediated by the mouse MAb 17-1A (Fig. 7, Table 3). However, Show et al (21) could not find any difference between mouse and chimeric MAb 17-1A in ADCC which might be explained by the short time (4h) ^{51}Cr release assay they used. To mediate lysis of target cells in vitro in ADCC monocytes/macrophages need a maturation period (4, 7, 9, 24) and thus, a 4h test might not be sufficient. Steplewski et al (9) have also shown that chimeric MAb 17-1A of the IgG_1 subclass mediated the strongest cytotoxicity while chimeric MAb 17-1A of IgG_4, IgG_3 and IgG_2 subclasses respectively was less effective in that order. The Fc part of the chimeric MAb binds probably with a higher affinity to the Fc-receptors on the effector cells than the mouse MAb. The aspargine-linked carbohydrate structures of the IgG molecule are directly involved in different biological functions,

such as binding to Fc receptors on macrophages/monocytes, activation of complement, and ADCC activity. Differences in the ability of IgG subclasses to participate in ADCC might be related to glycosylation at the CH_2 region. It is highly probable that the carbohydrate sequences of human MAbs would be more reactive with human Fc receptors than those of mouse MAbs (25). The same difference between a mouse MAb and a chimeric MAb in an ADCC system has also been described for L6, a MAb directed against adenocarcinoma cells, and MAb against CD20 on B cells (26,27). This in vitro finding might be of relevance for MAb therapy in human. Thus, treatment with chimeric MAb may be more effective than mouse MAb in the lysis of tumor cells. The generation of anti-mouse constant Ig antibodies is abolished but the induction of anti-idiotypic antibodies preserved, which might be of therapeutic value (28).

ACKNOWLEDGEMENT

This study was supported by grants from Cancer Society in Stockholm, the King Gustaf V Jubilee Found and the Swedish Cancer Society. For the generous gift of the monoclonal antibodies, we thank Dr H. Koprowski and Dr Z. Steplewski. We thank Miss Marie Karlsson for excellent secretarial assistance.

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Received for publication May 6, 1988
Accepted after revisions July 26, 1988